PATENT
Attorney Docket **044508-5023-US**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Christopher C. Broder *et al.***

Application No. **10/528,800**

Filed: **March 31, 2006**

Art Unit: **1648**

Examiner: **Benjamin P. Blumel**

Confirmation No. **9160**

For: **Compositions and Methods for the Inhibition of Membrane Fusion by Paramyxoviruses**

DECLARATION UNDER 37 C.F.R. 1.131

We, Christopher C. Broder and Katharine A. Bossart declare as follows:

1. We understand that the pending claims of the above-referenced application are directed to methods of inhibiting fusion between a membrane of a paramyxovirus and a plasma membrane of a cell, method of treating an infection of a virus and methods of treating or preventing infection by a paramyxovirus by administrating compositions contain polypeptides comprising SEQ ID NO: 1 or SEQ ID NO: 2 (*i.e.* synthetic peptides derived the heptad portion of Hendra and Nipah virus F protein). We also understand that the '800 application is a U.S. National Stage Application of PCT application PCT/US02/36283, filed on November 13, 2002 which claims priority to U.S. Provisional Application 60/331,231 filed on November 13, 2001.

2. The journal publication entitled "Functional Expression and Membrane Fusion Tropism of the Envelope Glycoproteins" published in the November 2001 issue of *Virology*, volume 290, pages 121 to 135 (a copy of which is attached as Exhibit A) was co-authored by Katharine N. Bossart, Lin-Fa Wang, Bryan T. Eaton and Christopher C. Broder. This issue of *Virology* was published online on November 15, 2001 and mailed to subscribers on November 20, 2001. As evidence of the publication date, we have submitted herewith an e-mail from the publisher confirming the publication date and the date on which the publication was available online (a copy of which is attached as Exhibit B).

3. The journal article in *Virology* reflects our work and the article evidences in part the subject matter of the pending claims. Our publication in *Virology* was published less than one year prior to the

International filing date of PCT application PCT/US02/36283 but after the filing of U.S. Provisional Application 60/331,231. Lin-Fa Wang; and Bryan T. Eaton, the other authors of the journal publication, were working under our direction and did not contribute to the conception of the invention, and hence, it is our understanding that, they are therefore not inventors of the above-referenced application nor International application PCT/US02/36283.

4. We have read the Office Action in the above-referenced application. We understand that the Examiner has cited this *Virology* publication, our own work, as alleged prior art to the pending claims. We also understand that the currently pending claims are directed to methods of preventing paramyxovirus infections with compositions containing peptides of SEQ ID NO: 1 or SEQ ID NO: 2 (*i.e.*, HeV-FC2 and NiV-FC1 peptides (*see* page 24, Table of the '800 application)).

5. This subject matter was invented in the United States before November 13, 2001 and published less than one year prior to the filing date of PCT application PCT/US02/36283. The subject matter was therefore invented prior to the date of publication of the journal as evidenced by the date of publication (earliest possible publication date of November 15, 2001).

6. We conceived the claimed invention in the United States prior to March 23, 2000. Evidence of the prior conception and reduction to practice is, for example, seen in U.S. Provisional Application 60/331,231 filed on November 13, 2001. Specifically, the provisional application is directed research discussed in the *Virology* article.

7. From before the filing of the provisional to the filing of the PCT application, we worked diligently on the invention. For example, as evident from the PCT application, we designed, isolated, and sequenced the specific heptad portions of Nipah and Hendra virus.

8. It is our understanding that since our *Virology* article was published less than one year from the filing of PCT application PCT/US02/36283, which supports the disclosure of the pending claims, our *Virology* article is not prior art to the pending claims under U.S. Patent law.

9. We further declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful

false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

Respectfully submitted,

Christopher C. Broder — Date

Katharine A. Bossart — Date 09/15/08

Exhibit A

Virology 280, 121–135 (2001)
doi:10.1006/viro.2001.1158, available online at http://www.idealibrary.com on IDEAL®

Functional Expression and Membrane Fusion Tropism of the Envelope Glycoproteins of Hendra Virus[1]

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Received July 9, 2001; returned to author for revision August 3, 2001; accepted August 22, 2001

Hendra virus (HeV) is an emerging paramyxovirus first isolated from cases of severe respiratory disease that fatally affected both horses and humans. Understanding the mechanisms of host cell infection and cross-species transmission is an important step in addressing the risk posed by such emerging pathogens. We have initiated studies to characterize the biological properties of the HeV envelope glycoproteins. Recombinant vaccinia viruses encoding the HeV F and G open reading frames were generated and glycoprotein expression was verified by metabolic labeling and detection using specific antisera. Glycoprotein function and cellular tropism were examined with a quantitative assay for HeV-mediated membrane fusion. Fusion specificity was verified through specific inhibition by anti-HeV antiserum and a peptide corresponding to one of the α-helical heptad repeats of F. HeV requires both F and G to mediate fusion. Permissive target cells have been identified, including cell lines derived from cat, bat, horse, human, monkey, mouse, and rabbit. Fusion negative cell types have also been identified. Protease treatments of the target cells abolished fusion activity, suggesting that the virus is employing a cell-surface protein as its receptor.

Key Words: Paramyxovirus; Hendra virus; Nipah virus; envelope glycoprotein; fusion; tropism; receptor.

INTRODUCTION

Fusion of the membrane of enveloped viruses with the plasma membrane of a receptive host cell is a prerequisite for viral entry and infection and an essential step in the life cycle of all enveloped viruses. The paramyxoviruses are negative-stranded RNA-containing enveloped viruses encompassing a variety of important human and animal pathogens including measles virus (MeV), canine distemper virus (CDV), human parainfluenza viruses (hPIV) 1–4, respiratory syncytial virus (RSV), and simian virus 5 (SV5) (reviewed in Lamb and Kolakofsky (2001)). These viruses contain two principal membrane-anchored glycoproteins that appear as spikes projecting from the envelope membrane of the viral particle when imaged in the electron microscope. One glycoprotein is associated with virion attachment to the host cell and, depending on the particular paramyxovirus, has been designated as the hemagglutinin–neuraminidase protein, the hemagglutinin protein (H), or the G protein, which has neither hemagglutinating nor neuraminidase activities. The second glycoprotein is the fusion protein (F), which facilitates the membrane fusion event between the virion and the host cell during virus infection (reviewed in Baker *et al.* (1999); Lamb (1993)). Following virus attachment to a permissive host cell, fusion at neutral pH between the virion and the plasma membranes ensues, resulting in delivery of the nucleocapsid into the cytoplasm (reviewed in Lamb and Kolakofsky (2001)). In a related process, cells expressing these viral glycoproteins at their surfaces can fuse with receptor-bearing cells, resulting in the formation of multinucleated giant cells (syncytia). The paramyxovirus F glycoprotein shares several features with other viral membrane fusion proteins, including the envelope glycoprotein of retroviruses like gp120/gp41 of HIV-1 and hemagglutinin of influenza virus (reviewed in Hernandez *et al.* (1996)). The biologically active F protein consists of two disulfide-linked subunits, F_1 and F_2, that are generated by the proteolytic cleavage of a precursor polypeptide known as F_0 (Klenk and Garten, 1994; Scheid and Choppin, 1974). All paramyxoviruses studied to date, with the exception of SV5, require both the attachment and F glycoproteins for membrane fusion (Paterson *et al.*, 1997). A precise understanding of how the attachment and fusion glycoproteins of the paramyxoviruses function in concert in mediating this membrane fusion process has yet to be

[1] The views expressed in this article are solely those of the authors, and they do not represent official views or opinions of the Department of Defense or The Uniformed Services University of the Health Sciences. K. N. Bossart performed this work as partial fulfillment of the requirements of the Ph.D. program in Microbiology and Immunology of the Uniformed Services University of the Health Sciences.

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elucidated. Although evidence of physical interactions has only been rarely detected, it is hypothesized that the binding protein must somehow signal and induce a conformational change in F leading to virion/host cell membrane fusion (Lamb, 1993).

In 1994, a new paramyxovirus, now called Hendra virus (HeV) and recognized to be a member of the subfamily *Paramyxovirinae*, was isolated from fatal cases of respiratory disease in horses and humans and was shown to be distantly related to MeV and other members of the *Morbillivirus* genus (Murray *et al.*, 1995). The outbreak occurred in the Brisbane suburb of Hendra and resulted in the death of 13 horses and their trainer and the nonfatal infection of a stablehand and a further 7 horses. At approximately the same time, in an unrelated incident ~100 km north of Hendra, a 36-year-old man experienced a brief aseptic meningitic illness after caring for and assisting at the necropsies of two horses subsequently shown to have died as a result of HeV infection, and 13 months later the man suffered severe encephalitis characterized by uncontrolled focal and generalized epileptic activity (O'Sullivan *et al.*, 1997). Subsequent to these events, an outbreak of severe encephalitis in people with close contact exposure to pigs in Malaysia and Singapore occurred in 1998 (Centers for Disease Control and Prevention, 1999). In all, more than 265 cases of encephalitis, including 105 deaths, had been reported in Malaysia, and 11 cases, with one death, were reported in Singapore. This may represent a near 40% fatality rate upon infection, because the incidence of subclinical human infections during these episodes has not been well defined. Follow-up studies indicated that this virus was most closely related to HeV and was named Nipah virus (NiV) after the small town in Malaysia from which the first isolate was obtained from a fatal human case (Chua *et al.*, 1999, 2000; Goh *et al.*, 2000; Lee *et al.*, 1999; Lim *et al.*, 2000). HeV and NiV are now two representatives of a new genus within the *Paramyxovirinae* subfamily called *Henipavirus* (Wang *et al.*, 2001; Wang and Eaton, in press).

Both HeV and NiV are unusual among the paramyxoviruses in their ability to infect and cause potentially fatal disease in a number of host species, including humans, and in that they have an exceptionally large genome. Understanding the mechanisms of how viruses like these emerge, mediate host cell infection, or undergo cross-species transmission is an important step toward determining how to address such emerging infectious disease threats. Our approaches to examine HeV have begun with the characterization of the virus' envelope glycoproteins, which facilitate the attachment and fusion events during infection. HeV possesses a F glycoprotein that likely mediates membrane fusion, and the attachment glycoprotein has been designated G. Here we report the development of a recombinant system to study HeV using recombinant vaccinia viruses encoding the F and G open reading frames (ORFs). HeV F and G expression was verified by metabolic labeling and detection using specific antisera. The cellular tropism of HeV was examined using a quantitative assay for HeV-mediated fusion. Fusion specificity was also verified through specific inhibition with a peptide corresponding to one of the α-helical heptad repeats of F and rabbit anti-HeV serum, which could specifically inhibit the fusion assay. HeV requires both F and G to mediate fusion. Permissive target cells have been identified, including those derived from cat, bat, horse, human, simian, mouse, and rabbit. Putative receptor negative cell types have also been identified, and protease treatments of target cells abolished fusion activity, suggesting that the virus is employing a cell-surface protein as its receptor.

RESULTS

Expression of Hendra virus F and G glycoproteins

HeV is classified as a zoonotic, biosafety level 4 (BSL-4) agent and thus its manipulation under laboratory conditions is highly restricted. To readily examine the biochemical and functional properties of the virus' envelope glycoproteins, the viral proteins responsible for host cell attachment and virion entry, we employed the vaccinia virus-based recombinant expression system (Broder and Earl, 1999; Carroll and Moss, 1997). For the production of recombinant-expressed HeV envelope glycoproteins, the HeV F and G ORFs (Gould, 1996; Wang *et al.*, 2000; Yu *et al.*, 1998) were subcloned into the vaccinia virus promoter driven expression vector pMC02 (Carroll and Moss, 1995) and recombinant viruses were prepared using standard techniques as detailed under Materials and Methods. In order to develop reagents to biochemically detect the HeV envelope glycoproteins, anti-F and anti-G peptide-specific rabbit antisera were prepared (see Materials and Methods). These antisera were titered using a peptide-specific ELISA and specificity was further cross-analyzed by competition ELISA. The HeV envelope glycoproteins F and G were produced in cell culture either by transient transfection with the appropriate plasmid construct or by infection with recombinant vaccinia virus. Shown in Fig. 1A is recombinant vaccinia virus-expressed HeV F immunoprecipitated with peptide-specific (anti-F_2 or anti-G) and virus-specific antisera. The vaccinia-expressed HeV F appeared predominantly as the uncleaved precursor protein, F_0, and as the processed F_1 subunit. The F_2 subunit (~19 kDa) was not readily detected under these conditions, likely due to a combination of the amount and specific activity of the metabolically labeled polypeptide. This profile of the HeV F was quite similar to that of several other paramyxovirus F glycoproteins (Bagai and Lamb, 1995; Nussbaum *et al.*, 1995; Yao *et al.*, 1997), with apparent molecular masses for F_0 of ~61 kDa and F_1 ~49 kDa, and identical to the F polypeptides derived from purified HeV particles (Michal-

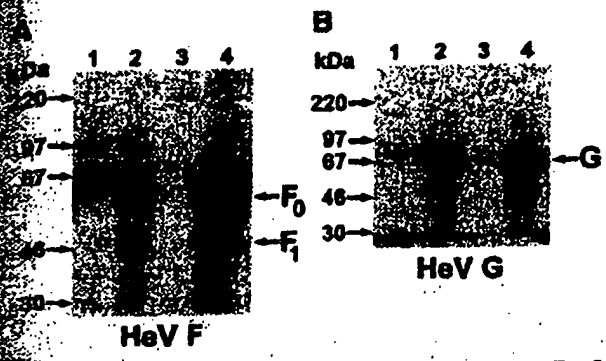

FIG. 1. Expression of recombinant HeV F and G glycoproteins. The F and G glycoprotein ORFs were subcloned into vaccinia virus promoter-driven expression vector pMC02 (Carroll and Moss, 1995). HeLa cells were infected with HeV F or G encoding recombinant vaccinia viruses and incubated for 16 h at 37°C. Beginning at 6 h postinfection, the cells were labeled overnight with [^{35}S]methionine/cysteine. Lysates were prepared in buffer containing Triton X-100 and clarified by centrifugation. Immunoprecipitation was performed with rabbit polyclonal antiserum against synthetic F_2 or G-specific peptides or rabbit anti-HeV antiserum (see Materials and Methods), followed by Protein G–Sepharose. The radiolabeled proteins were resolved by 10% SDS–PAGE under reducing conditions and detected by fluorography. (A) HeV F immunoprecipitated lysates; lanes 1 and 2 with rabbit anti-HeV antiserum, lanes 3 and 4 with rabbit anti-F_2 peptide antiserum. (B) HeV G immunoprecipitated lysates; lanes 1 and 2 with rabbit anti-HeV antiserum, lanes 3 and 4 with rabbit anti-G peptide antiserum. Lanes 1 and 3 of each panel are precipitates prepared from lysates of cells infected with a control vaccinia virus.

ski *et al.*, 2000; Murray *et al.*, 1998; Wang, 1998). Shown in Fig. 1B is metabolically labeled recombinant vaccinia virus-expressed HeV G immunoprecipitated with rabbit anti-peptide and anti-HeV antisera. The vaccinia-expressed HeV G possessed an apparent molecular mass of ~75 kDa, also quite similar to HeV G derived from purified HeV virions (Murray *et al.*, 1998; Wang *et al.*, 1998), as well as to the H proteins from MeV and CDV (Nussbaum *et al.*, 1995). The profiles of the vaccinia-expressed F and G glycoproteins observed using rabbit anti-HeV antiserum were identical to those obtained with the peptide-specific antisera; compare lanes 2 and 4 in each panel of Fig. 1.

Syncytia formation mediated by HeV envelope glycoproteins

To evaluate whether the F and G ORFs encoded functional HeV envelope glycoproteins, it was necessary to demonstrate their ability to mediate membrane fusion. In initial experiments, the plasmids containing either HeV F or G were transfected alone or in combination into several cell lines that included murine 3T3, human 293T, simian BSC-1, human TK$^-$, and human HeLa cells. When the HeV F or HeV G constructs or the plasmid vector control was transfected into cells individually, no syncytia formation was evident in any of several cell lines examined (not shown). However, when the HeV F and G plasmid constructs were cotransfected, syncytia formation was evident in all cell lines tested with the exception of HeLa (not shown), with some variation noted in the average size of individual syncytia among the different cell types over the same incubation period. Since HeLa cells were unable to support syncytia formation the presumption was that these cells lack a functional HeV receptor and would not serve as permissive target cells for HeV-mediated fusion, thus they were chosen as the HeV F- and G-expressing effector cell populations for subsequent membrane fusion experiments. In so doing, HeV glycoprotein-expressing HeLa effector cells would not undergo spontaneous membrane fusion and would also likely prevent possible intracellular receptor/envelope glycoprotein complex formation, which might interfere in quantitative membrane fusion assessments. Shown in Fig. 2 are the results observed with recombinant vaccinia virus-expressed HeV glycoproteins in HeLa effector cell populations mixed with murine 3T3 cell targets as a representative example, where syncytia are evident only when F and G are coexpressed (Fig. 2D). Like most other paramyxoviruses that have been examined, HeV-mediated fusion and syncytia formation required the expression of both the F and G glycoproteins to mediate syncytia formation. These data demonstrate that the cloned ORFs for the predicted HeV F and G genes do encode functional glycoproteins.

Quantitation of HeV-mediated fusion

Although syncytia formation was evident, visual observation is only semiquantitative and possesses low sensitivity for measuring viral glycoprotein-mediated membrane fusion. Previously, we developed a functional reporter-gene system for the examination of the membrane fusogenic properties of the envelope glycoprotein of HIV-1 (Broder and Berger, 1995; Broder *et al.*, 1994; Nussbaum *et al.*, 1994). This system is based on gene expression using the recombinant vaccinia virus system (Berger *et al.*, 1995), where in addition to the viral envelope glycoproteins and viral receptors being expressed on effector and target cell populations, respectively, one cell population also expresses bacteriophage T7 RNA polymerase and the other a T7 promoter-driven *Escherichia coli LacZ* cassette (see Materials and Methods). Thus, cell–cell fusion results in the specific production of β-galactosidase (β-Gal), which can be quantified. This assay has proven especially useful in the study of envelope glycoproteins derived from viruses that employ a pH-independent mechanism of membrane fusion for virion entry (Alkhatib *et al.*, 1996b; Bagai and Lamb, 1995; Broder and Berger, 1995; Chung *et al.*, 1999; Feng *et al.*, 1996; Krueger *et al.*, 2001; Nussbaum *et al.*, 1995; Pastey and Samal, 1997; Santoro *et al.*, 1999; Takikawa *et al.*, 2000). Using this assay, HeV glycoprotein-expressing

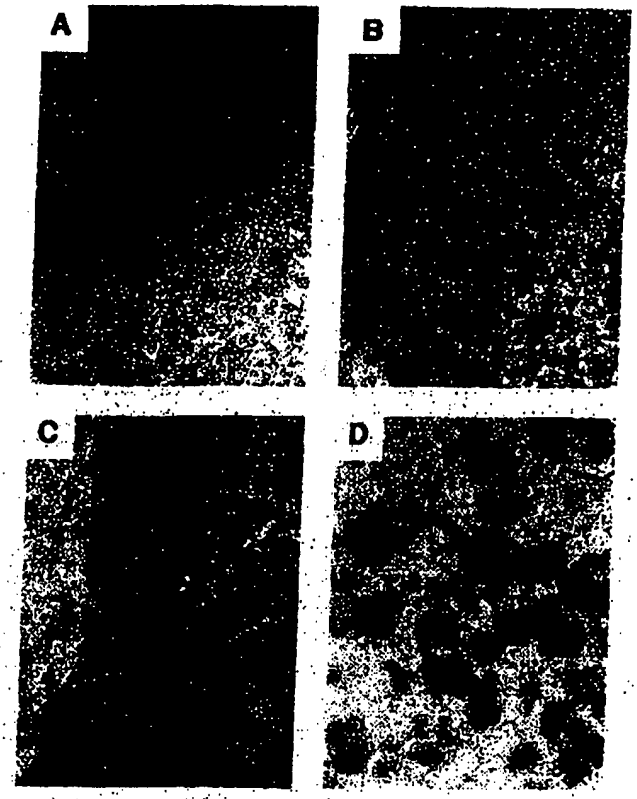

FIG. 2. Syncytia formation mediated by Hendra virus glycoprotein-expressing cells. HeLa cells were infected with vaccinia virus recombinants encoding HeV F (B), HeV G (C), both F and G glycoproteins (D), or a control vaccinia virus (A), along with a vaccinia virus recombinant encoding T7 RNA polymerase. Partner 3T3 cells were infected with the *E. coli LacZ*-encoding reporter vaccinia virus vC821R. The HeV glycoprotein-expressing cells (1×10^5) were mixed with the 313 partner cells (1×10^4) in duplicate wells of a 96-well plate and incubated at 37°C. After 3 h the cells were fixed and stained with crystal violet and photographs were taken at 400× magnification.

HeLa effector cells were prepared and mixed with various target cell populations. Typically, the target and effector cell populations are assessed in duplicate or triplicate in 96-well plate format and incubated for 2–4 h following mixing. Cell lysates are prepared and processed for β-Gal quantification. The results shown in Fig. 3 are the HeV-mediated fusions measured in the same series of cell lines that was examined in the syncytia formation assay. Some differences in fusion activity as measured by the level of β-Gal activity were observed between those cell lines that were syncytia positive, with the mouse 3T3 target cells consistently yielding the highest levels. HeLa target cells were again negative for HeV-mediated fusion, thus corroborating the syncytia assay results, and since this cell-fusion reporter gene assay is independent of syncytia formation, it appears that HeLa cells are indeed fusion nonpermissive. It is also evident from this experiment that efficient HeV-mediated membrane fusion requires the presence of both the F and the G glycoproteins.

Specificity of HeV-mediated fusion activity

To further assess the specificity and utility of the HeV-mediated fusion system we sought ways to specifically inhibit the cell-fusion process. There have been considerable advances in the understanding of the structural features and development of mechanistic models of how several viral envelope glycoproteins function in driving the membrane fusion reaction (reviewed in Chan and Kim (1998); Skehel and Wiley (1998); Weissenhorn *et al.* (1999)). One important feature of many of these fusion glycoproteins is the presence of two α-helical domains, referred to as heptad repeats, that are involved in the formation of a trimer-of-hairpins structure (Hughson, 1997; Singh *et al.*, 1999). These domains are also referred to as the amino (N)-terminal and the carboxyl (C)-terminal heptad repeats, and peptides corresponding to either of these domains can inhibit the activity of the viral fusion glycoprotein when present during the fusion process. Here we noted two putative α-helical domains in the HeV F glycoprotein analogous to the heptad repeats present in SV5 F. One HeV F heptad domain is proximal to the fusion peptide of F_1 (N-terminal heptad repeat), and the other is very close to the predicted transmembrane domain of F_1 (C-terminal heptad repeat) (Fig. 4A). Helical wheel analysis revealed a high degree of sequence homology with the important functional residues of the SV5 heptad repeats (Fig. 4C) (Joshi *et al.*, 1998). To determine whether these structures play a similar important role in HeV fusion, a 42-amino-acid peptide (FC1) derived from the C-terminal heptad repeat (Fig. 4B) was synthesized and tested for its ability to interfere with HeV-mediated fusion. An available nonspecific 44-amino-acid peptide derived from the cytoplasmic tail of the interleukin-2 (IL-2) receptor gamma chain was used as an irrelevant peptide control. Shown in Fig. 5 are the results of HeV-mediated fusion obtained in the presence of a series of FC1 peptide, or irrelevant control peptide, concentrations. The FC1 peptide could potently inhibit fusion in a dose-dependent manner and was completely inhibitory in the nanomolar range, with an IC_{50} of 4.4 nM. These data strongly suggest that the HeV fusion mechanism is likely highly analogous to other viral fusion systems where a trimer of hairpins has been hypothesized to form. The specific inhibition of the HeV-mediated fusion assay by a synthetic peptide that targets the F glycoprotein further demonstrates the specificity of this HeV-mediated cell fusion assay.

To explore the utility of the HeV fusion assay, we tested the ability of HeV-specific antiserum to inhibit fusion. Rabbit anti-HeV antiserum was serially diluted and added to HeLa effector cells expressing HeV F and G glycoproteins. Target cells were immediately added

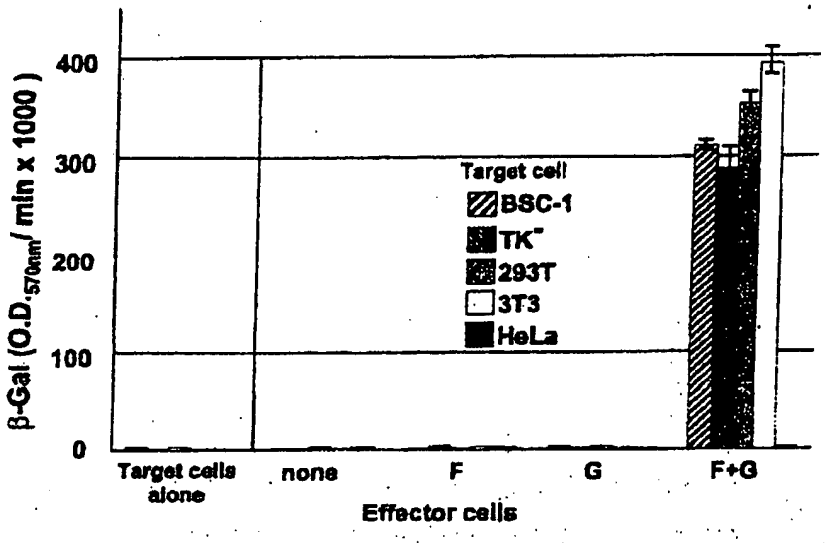

FIG. 3. Quantitation of HeV-mediated cell fusion. HeLa cells were infected with vaccinia recombinants encoding HeV F, HeV G, both F and G glycoproteins, or none, along with a vaccinia recombinant encoding T7 RNA polymerase (effector cells). Each designated target cell type was infected with the *E. coli lacZ*-encoding reporter vaccinia virus vCB21R. The HeV glycoprotein-expressing cells (1×10^5) were mixed with each target cell type (5×10^5) in duplicate wells of a 96-well plate. After 3 h at 37°C, Nonidet-P40 was added and β-Gal activity was quantitated. The level of background β-Gal activity in target cell populations alone is indicated. The β-Gal activity from target cells mixed with HeLa partner cells infected with only T7 RNA polymerase-encoding vaccinia virus and no vaccinia recombinants encoding HeV glycoproteins is indicated as "none."

and mixed, and fusion was allowed to proceed for 3 h (see Materials and Methods). Shown in Fig. 6 are the HeV fusion results obtained in the presence of HeV-specific rabbit antiserum in comparison to normal rabbit serum. There was little nonspecific inhibitory activity exhibited by the normal rabbit sera and was maximally ~15%, at the highest sera concentrations (data not shown), whereas inhibition by the HeV-specific antiserum could block the fusion assay by >90% at a 1:50 dilution and there was approximately 50% inhibition at a 1:200 dilution. These results were quite significant in light of the high levels of expressed HeV F and G envelope glycoproteins on the surfaces of the HeLa effector cells, and the potency of inhibition observed here may be related to the requirement of two envelope glycoproteins in the HeV fusion process and the polyclonal nature and characteristics of the antibodies present. Indeed, the peptide-specific anti-F_2 and anti-G rabbit sera developed here had no inhibitory effect when examined (not shown), which is perhaps not unexpected since it is highly likely that peptide-specific antisera would contain no conformation-dependent antibody reactivities and be reactive to only a limited subset of epitopes, unlike serum derived from whole HeV-immunized rabbits.

Protease treatment of target cells destroys HeV-mediated fusion activity

The utility of the HeV-mediated fusion assay combined with the observation that cell lines derived from the same species may be either permissive or resistant to fusion prompted an examination of the nature of the unknown HeV receptor, and permissive target and effector cells were subjected to various pretreatments prior to their use in the cell fusion assay. HeV contains neither hemagglutinating nor neuraminidase activity (Murray *et al.*, 1995), suggesting that the cellular receptor may not be sialic acid (Yu *et al.*, 1998). We treated effector cells with excess sialic acid over a range of concentrations and in no case was any inhibitory effect observed on HeV-mediated fusion (data not shown). We have also shown that neuraminidase treatment of Vero cells does not inhibit HeV infection but can abrogate their susceptibility to newcastle disease virus (NDV) and influenza virus A, two viruses that employ sialic acid as receptors (B. T. Eaton, unpublished results). Together, these data are in agreement with the notion that the HeV G protein is not employing sialic acid moieties as receptors. However, protease treatment of target cells with increasing doses of either proteinase K or trypsin resulted in significant decreases in subsequent HeV-mediated fusion in a dose-dependent manner (Fig. 7). The absolute values of β-Gal reporter activity are different between the two conditions because these are independent experiments. Together, these results support the hypothesis that HeV, like one of its most closely related family members, MeV, is employing a surface-expressed protein as a functional receptor for attachment and fusion.

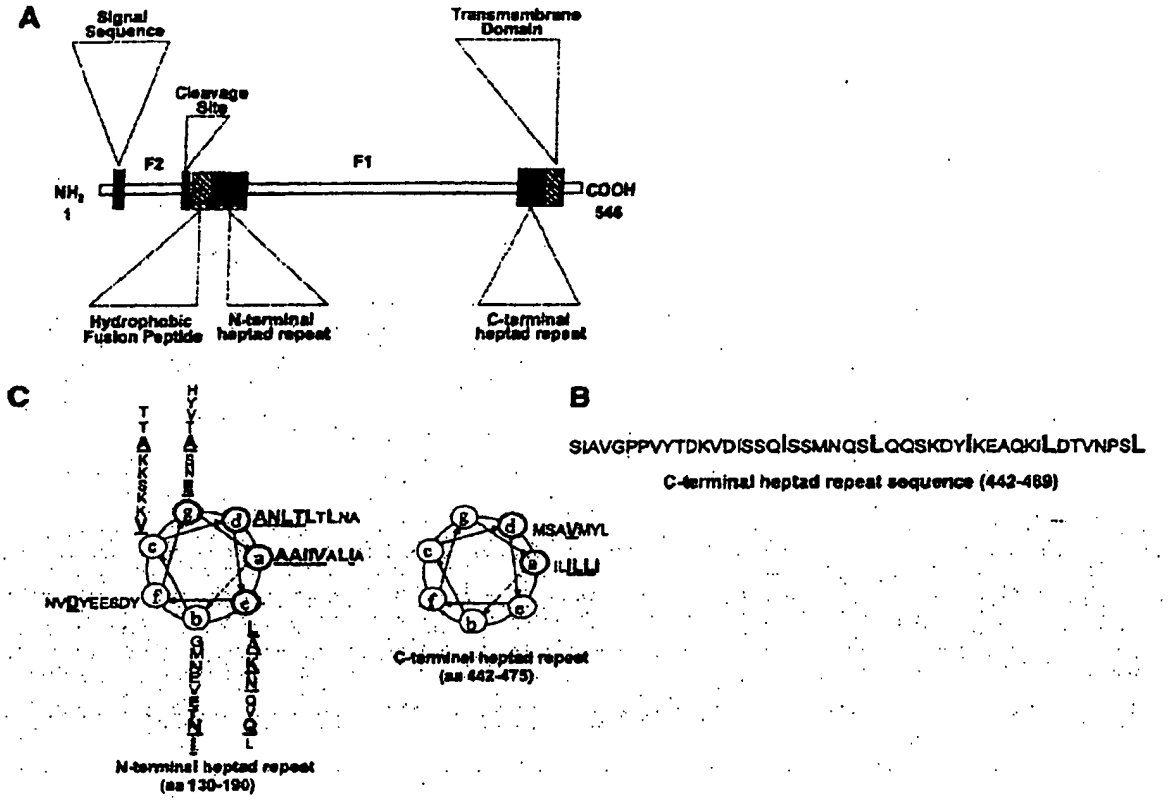

FIG. 4. Location of HeV F glycoprotein heptad repeats. (A) Diagram of the HeV F glycoprotein depicting important structural and functional elements. (B) Amino acid sequence of the C-terminal heptad repeat of HeV F; boldface and enlarged amino acids represent the important hydrophobic residues that are capable of forming a leucine zipper. (C) Helical wheel representation of the N- and C-terminal heptad repeats of HeV F. Boldface points on the helical wheel indicate important residue locations on the helix structure of the F protein of SV5 that mediate protein–protein interactions (Joshi *et al.*, 1998). Point a of one N-terminal heptad is thought to interact with point d of another SV5 F N-terminal heptad in a parallel orientation. Point e of the N-terminal heptad is thought to interact with point a of the C-terminal heptad, and point g of the N-terminal heptad is believed to interact with point d of the C-terminal heptad in an antiparallel orientation. In all, it is hypothesized that three N-terminal heptad repeats and three C-terminal heptad repeats of three SV5 F proteins mediate the necessary protein–protein interactions that stabilize the fusogenic SV5 F trimer formation. Enlarged underlined amino acids represent HeV F residues that are identical to those found in the N- and C-terminal heptad repeats of SV5; enlarged but not underlined amino acids are hydrophobic conservative substitutions in HeV F as compared to SV5 F.

Species tropism of HeV-mediated cell fusion

Unlike other paramyxoviruses, HeV has been clearly implicated in cross-species infections, including human, that can result in significant morbidity and mortality. Using the HeV-mediated cell fusion system developed here, we examined the target cell species tropism of HeV using a battery of available cell lines and primary cultures. Shown in Fig. 8, are the cell fusion results obtained with a series of alternate animal species cell lines. Target cells derived from an insectivorous bat, cells from horse (the first animal to contract HeV disease), and human TK$^-$ cells were all capable of permissive fusion with cells expressing the HeV F and G glycoproteins. Cat embryo cells were also permissive targets for HeV-mediated fusion, and cats have been shown to be highly susceptible to HeV infection, manifesting pathology very similar to that observed in naturally and experimentally infected horses (Hooper *et al.*, 2001). These results illustrate that the cell fusion tropism demonstrated by our functional recombinant assay parallels natural and experimental HeV infections. Other cells that were negative for fusion were pig kidney and duck embryo cell lines, as well as primary chick embryo fibroblasts. Although rabbits, monkeys, and mice have not been shown to be infected by HeV in nature, it is of interest that these cells also express the HeV receptor. The susceptibility of rabbits to HeV infection remains to be verified. In all cases, both HeV F and G together were required to mediate efficient fusion with all permissive target cell populations (Fig. 8). Finally, because both permissive and nonpermissive human target cell lines have been identified, we chose to examine primary blood cell populations of human peripheral blood lymphocytes (PBL) and macrophages. Both stimulated and unstimulated PBL and mac-

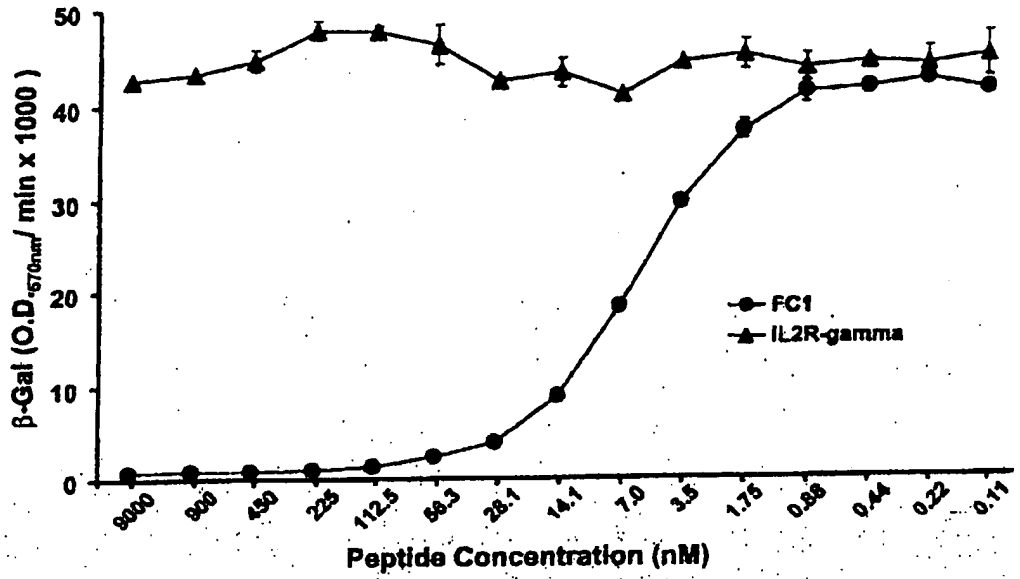

FIG. 5. Specificity of HeV-mediated fusion. HeLa cells were infected with vaccinia recombinants encoding the HeV F and G glycoproteins along with a vaccinia recombinant encoding T7 RNA polymerase (effector cells). Human TK⁻ cells were infected with the *E. coli LacZ*-encoding reporter vaccinia virus vCB21R (target cells). Peptides were diluted and added to the HeV glycoprotein-expressing cells (1×10^5) in a 96-well plate; TK⁻ cells were then added (1×10^5). Each peptide concentration was performed in duplicate in 96-well plate format. After 3 h at 37°C, Nonidet-P40 was added and β-Gal activity was quantitated.

rophages were examined and compared to HeLa target cells; however, in no case was significant fusion observed (Fig. 9). It should be noted that the level of signal observed is extremely low and that the signal observed with HeLa cell targets is less than twice the vector control and not considered significant. The hypothesis

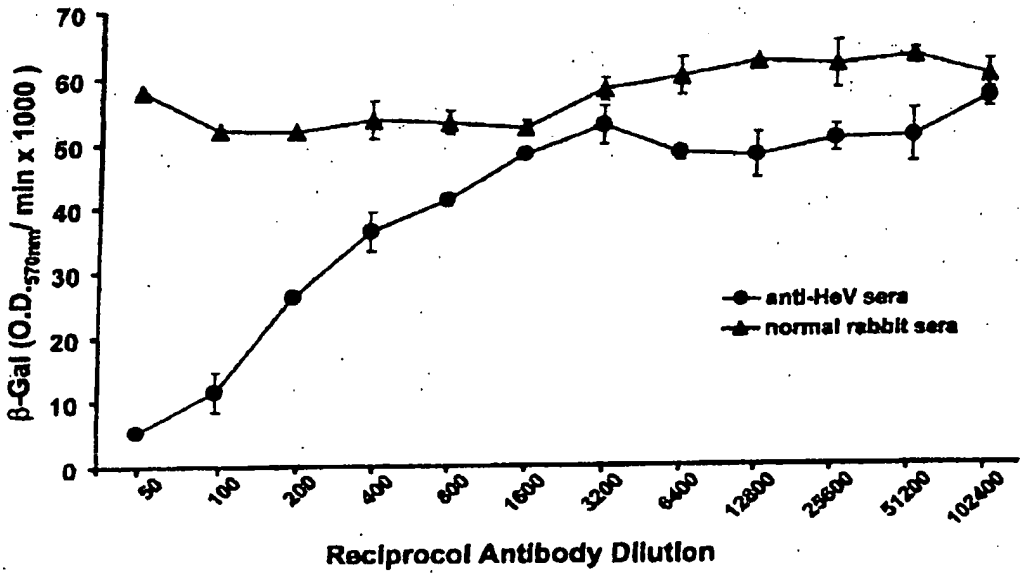

FIG. 6. HeV fusion and blocking by specific rabbit antiserum. HeLa and TK⁻ cells were prepared as described in the legend to Fig. 5. Rabbit sera were diluted and added to the HeV glycoprotein-expressing cells (1×10^5) in 96-well plate format and TK⁻ target cells were then added (1×10^5). Each serum dilution was performed in duplicate wells. After 3 h at 37°C, Nonidet-P40 was added and β-Gal activity was quantitated.

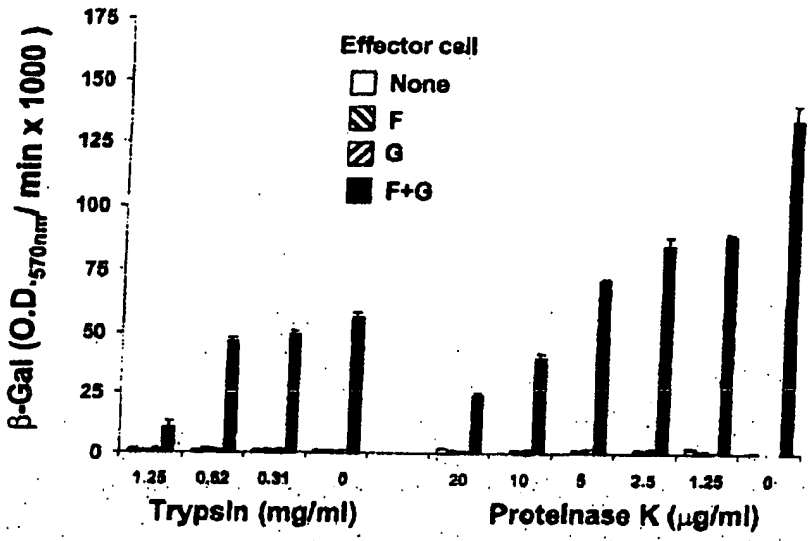

FIG. 7. Protease treatment of target cells destroys HeV-mediated fusion permissiveness. HeLa and TK⁻ cells were prepared as described in the legend to Fig. 6. TK⁻ target cells were treated with different concentrations of either trypsin or proteinase K for 2 min at room temperature, quenched with 10 ml of EMEM-10, washed once, and recounted. HeV glycoprotein-expressing cells (1×10^4) and protease-treated TK⁻ cells (1×10^4) were then mixed in 96-well plate format in duplicate. After 3 h at 37°C, Nonidet-P40 was added and β-Gal activity was quantitated. The data shown are results derived from independent experiments.

that HeV utilizes a cellular protein receptor in the process leading to fusion and syncytium formation as well as the fact that HeLa cells lack this receptor or at least a functional receptor, coupled with this highly sensitive and specific HeV-mediated fusion system, provides an avenue for receptor identification.

DISCUSSION

The results presented here have laid the groundwork for studying the fusion and attachment membrane glycoproteins of the newly emerging Hendra virus. The genetic and biological features of HeV as well as the

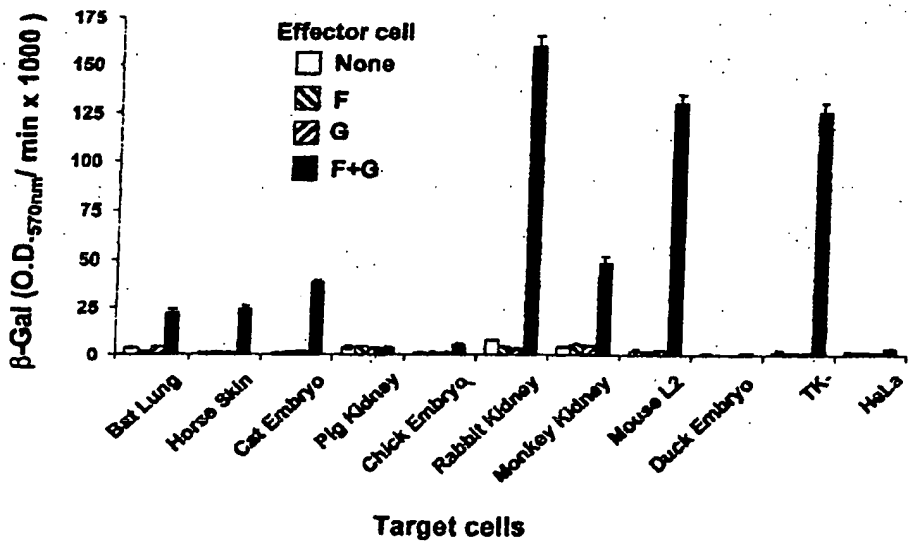

FIG. 8. Species tropism of HeV-mediated cell fusion. HeLa effector cells were prepared as described in the legend to Fig. 3. Each designated target cell type was infected with the *E. coli LacZ*-encoding reporter vaccinia virus vC821R. Cell populations were mixed and cell fusion was measured as described in the legend to Fig. 6.

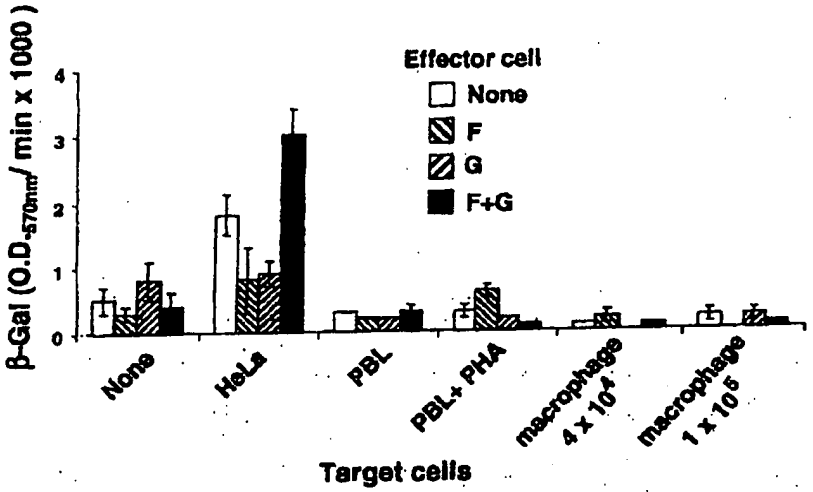

FIG. 9. Primary human cell tropism of HeV-mediated cell fusion. Human PBL and macrophage cultures were incubated overnight prior to use. Stimulated PBL were prepared by culturing cells with 3 μg/ml phytohemagglutinin for 3 days prior to use. HeLa effector cells were prepared as described in the legend to Fig. 3. Each designated target cell type was infected with the *E. coli lacZ*-encoding reporter vaccinia virus vCB21R. Cell populations were mixed and cell fusion was measured as described in the legend to Fig. 5.

related NiV have placed them into a new genus, *Henipavirus*, in the *Paramyxovirinae* subfamily (Eaton, 2001; Field *et al.*, 2001; Hooper *et al.*, 2001; Wang *et al.*, 2001, in press). Indeed, HeV and NiV are somewhat unique among the paramyxoviruses in their ability to cause severe and fatal disease in several animal species and humans. Understanding the mechanisms of how viruses like these can emerge, mediate host cell infection, and perform cross species transmission is an important step toward determining how to address new infectious disease threats such as these.

Here we report the development of a recombinant expression system to examine the membrane glycoproteins of HeV and describe several features of their functional activity. Similar results have been recently obtained in our laboratory with the membrane glycoproteins of NiV (Bossart and Broder, unpublished results). Radioimmunoprecipitation using anti-HeV peptide-specific sera and rabbit anti-HeV antiserum showed that recombinant vaccinia virus-expressed HeV F and G glycoproteins were comparable to the cognate proteins in purified virus (Murray *et al.*, 1998). The molecular mass of the F_0 precursor was ~61 kDa and the processed F_1 subunit ~49 kDa. The F_2 subunit, ~19 kDa, was not observed biochemically using our available reagents. The failure of F_2 detection by either antiserum could be due to the paucity of F_2 antibodies, but is more likely related to assay sensitivity. Nevertheless, F_2 is present based on the functionality of HeV F glycoprotein in fusion and the fact that the anti-peptide antiserum used is specific for an F_2 sequence. In addition, we have observed a similar lack of F_2 detection in radioimmunoprecipitation of functional MeV and CDV F glycoproteins (Nussbaum *et al.*, 1996). The recombinant-expressed G glycoprotein was ~75 kDa. Both these glycoproteins have several predicted N-linked glycosylation sites (Michalski *et al.*, 2000) and studies are under way to examine which sites are utilized and are important for biological function. Based on the similarity in molecular weights in comparison to other members of the *Paramyxoviridae* the F and G glycoproteins of HeV are undoubtedly N-glycosylated at one or more sites.

The functionality of the HeV F and G glycoproteins was immediately apparent through recombinant expression and the appearance of syncytia among cell populations expressing both HeV F and G glycoproteins. Indeed, the HeV glycoproteins were functionally expressed in pilot experiments prior to the availability of antiserum for biochemical detection. By adapting our cell–cell fusion reporter-gene assay to the HeV-mediated fusion system we were able to systematically examine, in a quantitative manner, a battery of target cell populations representing a variety of cell types and animal species. In so doing, and more importantly for the development of strategies to identify the receptor utilized by HeV, several cell lines and some primary cell types were found to be likely receptor-negative for HeV. Among these possible receptor-negative cell types were the human HeLa cell line and primary human PBL and macrophages. It was somewhat surprising that pig cells were negative, in light of the observation that pigs are a natural target of the related NiV in nature. The examination of additional pig cell lines and or primary cells will be undertaken when they become available. In general, however, the detection of HeV-mediated fusion correlated well with those animal species known to be permissive for experimental

HeV infection, such as horse, cat, and bat. Although we examined an insectivorous bat cell line, the natural reservoir of HeV appears to be a frugiverous bat. Recently it was found that approximately 50% of Australian fruit bats, commonly known as flying foxes, have antibodies to HeV, and HeV-like viruses have been isolated from bat uterine fluids; it appears that these animals are the natural host for the virus (Field *et al.*, 2001; Halpin *et al.*, 1999, 2000; Young *et al.*, 1996). Finally, the generation of very high titers of anti-HeV antibodies following administration of a single dose of HeV to rabbits suggests that such animals may be susceptible (Westbury *et al.*, 1995) and the susceptibility of rabbit kidney cells to HeV-mediated fusion is consistent with this suggestion. However, some caution must be exercised in correlating *in vivo* susceptibility to infection with the ability to form syncytia in an *in vitro* system. For example, the capacity of mouse L2 cells to form syncytia contrasts with the failure of HeV to infect BALB/c mice by intranasal or parental routes (B. T. Eaton, unpublished results).

Until recently, MeV, a morbillivirus and one of the viruses most closely related to HeV and NiV, was the only paramyxovirus shown to be capable of employing a cell-surface protein as functional receptor. MeV vaccine strains, in contrast to field isolates, can efficiently employ CD46 as a functional receptor (Naniche *et al.*, 1993) and MeV H glycoprotein has been shown to specifically complex with CD46 (Nussbaum *et al.*, 1995). Further, MeV field isolates as well as vaccine strains have recently been shown to be capable of utilizing signaling lymphocyte activation molecule (SLAM; CD150) (Tatsuo *et al.*, 2000, 2001). SLAM is also capable of serving as a receptor for several other morbilliviruses (Tatsuo *et al.*, 2001). Here we assessed the nature of the unknown HeV cellular receptor using the cell–cell fusion assay and found that protease treatment of permissive target cells inhibits HeV-mediated membrane fusion. Similar experiments in the characterization of HIV-1 envelope-mediated cell–cell fusion had also demonstrated this inhibition, where the CD4 receptor is readily removed from the cell surface by trypsin (Broder *et al.*, 1993). We hypothesize that since the HeV attachment glycoprotein has neither hemagglutinin nor neuraminidase activities, that protease treatment prevents fusion of an otherwise permissive target cell, and that certain cell lines from the same species can be clearly positive or negative for fusion, HeV is likely to utilize a surface-expressed protein receptor for virus entry and infection. The cell–cell fusion system described here is ideally suited to use in an expression cloning strategy for identifying the HeV receptor as was successfully done to discover the first HIV-1 coreceptor (Feng *et al.*, 1996).

All viral membrane glycoproteins that are the mediators of membrane fusion, virion attachment, or both are invariably oligomeric (Doms *et al.*, 1993). Considerable advances in the understanding of the structural features of these oligomeric viral envelope glycoproteins have been attained in recent years and have centered on the influenza virus and HIV systems. A notable structural feature of many of these fusion glycoproteins is the presence of two α-helical domains referred to as heptad repeats (Chambers *et al.*, 1990; de Groot *et al.*, 1987) that are important for both oligomerization and function of the glycoprotein, where they are involved in the formation of a trimer-of-hairpins structure (Hughson, 1997; Singh *et al.*, 1999). Peptides corresponding to either of these domains can potently inhibit the fusion process, first noted with sequences derived from the gp41 subunit of HIV-1 envelope glycoprotein (Jiang *et al.*, 1993; Wild *et al.*, 1994). Inhibition of the formation of the trimer-of-hairpins structure inhibits the fusion process, and this mechanism has been modeled and described by several groups (Chan and Kim, 1998; Munoz-Barroso *et al.*, 1998; Rimsky *et al.*, 1998; Root *et al.*, 2001; Russell *et al.*, 2001). Indeed, the development and clinical application of fusion inhibitors, as antiviral therapies for HIV-1, have been a direct result of this area of research.

Recently, an α-helical trimeric core complex was defined in the F protein of SV5 and is also believed to be either the fusion competent structure or the structure formed after fusion has occurred, analogous to HIV-1 gp41 (Baker *et al.*, 1999; Dutch *et al.*, 1999; Lamb *et al.*, 1999). Similarly, the fusion protein core of RSV has also been defined (Zhao *et al.*, 2000). In addition, peptide sequences from the N-terminal and/or C-terminal heptads of the F glycoproteins of SV5, MeV, RSV, hPIV, NDV, and Sendai virus have been shown to be potent inhibitors of membrane fusion (Joshi *et al.*, 1998; Lambert *et al.*, 1996; Rapaport *et al.*, 1995; Wild and Buckland, 1997; Young *et al.*, 1997, 1999). Here we analyzed the heptad repeats of HeV F using helical wheel diagrams and identified the sequences that would be likely inhibitors of HeV-mediated fusion. We then examined the specificity of our recombinant HeV fusion system using a synthetic 42-amino-acid peptide (FC1) corresponding to the HeV F C-terminal heptad. The FC1 peptide could completely inhibit HeV-mediated fusion in the nanomolar range (IC_{50} of 4.4 nM). The FC1 peptide is currently being evaluated as an inhibitor of live HeV infection under BSL-4 conditions and may represent a therapeutic avenue for both HeV and NiV infections. Indeed, the HeV F C-terminal heptad peptide was also capable of inhibiting recombinant NiV-mediated fusion at slightly lower efficiencies, likely due to several mismatches in the heptad sequence (Bossart and Broder, unpublished results).

HeV cell–cell fusion was also characterized using HeV-specific rabbit antiserum, which could block the cell–cell fusion assay with considerable efficiency. In addition, NiV-specific rabbit sera could block HeV-mediated fusion at lower levels of efficiency (not shown) and this would be in agreement with the observed antigenic cross-reactivity seen with HeV and NiV (Daniels *et al.*,

2001). Because this assay can be performed under BSL-2 conditions and is highly adaptable to high-throughput screening, it may be a useful tool in the titering of neutralizing antiserum outside BSL-4 containment. Experiments are under way to make comparative assessments of this cell–cell fusion assay with live HeV and NiV neutralization assays.

In summary, we have established a recombinant system to express and characterize the HeV F and G membrane glycoproteins and study the HeV-mediated membrane fusion process. We have shown that efficient membrane fusion requires both the F and the G glycoproteins as is seen for almost all other paramyxoviruses. In addition, fusion can be specifically inhibited with either antiserum or targeted peptides, and to a significant degree fusion parallels observed and experimental HeV infection. We have also identified possible receptor-negative cell types. These preliminary studies have laid the foundation for numerous approaches to develop new reagents and to examine the many features of the fusion and attachment glycoproteins of this interesting and unique emerging paramyxovirus.

MATERIALS AND METHODS

Cell lines

The following cell lines were obtained from the American Type Culture Collection (ATCC): HeLa cells (ATCC CCL 2); BSC-1 (ATCC CCL 26); CV-1 (ATCC CCL 70); HuTK$^-$143B (TK$^-$) (ATCC CRL 8303); RK-13 (rabbit) (ATCC CCL 37); *Equus caballus* (horse) (ATCC CCL-57); *Sus scrofa* (pig) (ATCC CL-101); *Tadarida brasilliensis* (bat) (ATCC CCL-88). Mouse L2 cells were provided by Anthony Maurelli (Uniformed Services University, Bethesda, MD). Human PBL and macrophages were provided by Tzanko Stantchev (Uniformed Services University, Bethesda, MD). Primary chick embryo fibroblasts (CEF) were provided by Norman Cooper (National Institutes of Health, Bethesda, MD). 293T, 3T3, cat embryo, and duck embryo cell lines were provided by Jay A. Levy (University of California, San Francisco, San Francisco, CA).

Culture conditions

HeLa, mouse L2, 3T3, 293T, and human macrophage cell monolayers were maintained in Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, MD) supplemented with 10% cosmic calf serum (CCS) (HyClone, Logan, UT) and 2 mM L-glutamine (DMEM-10). BSC-1, CV-1, TK$^-$, and CEF cell monolayers were maintained in Eagle's minimal essential medium (EMEM) (Quality Biologicals) supplemented with 10% CCS and 2 mM L-glutamine (EMEM-10). Duck embryo, cat embryo, and human PBL were maintained in RPMI 1640 (Quality Biologicals) supplemented with 10% CCS and 2 mM L-glutamine (RPMI-10). Rabbit and horse cell monolayers were maintained in enriched EMEM (Quality Biologicals) supplemented with 10% CCS, 1 mM sodium pyruvate, and 2 mM L-glutamine. Bat cell monolayers were maintained in enriched EMEM containing 0.85 g/L sodium bicarbonate, 2 mM L-glutamine, and 10% CCS. Pig cell monolayers were maintained in Medium 199 (Quality Biologicals) containing 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, and 3% CCS. All cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Plasmids and recombinant vaccinia viruses

For expression of recombinant HeV F and G glycoproteins, the F and G glycoprotein ORFs were subcloned into the vaccinia virus promoter-driven expression vector pMCO2 (Carroll and Moss, 1995). The HeV F ORF was initially PCR amplified from plasmid pCP514 (HeV F gene in pFastBac1) (Gould, 1998; Wang *et al.*, 2000) using primers 5′-GTTTAAACGTCGACATGGCTACACAAGAGGTCAGG-3′ (KB1) and 5′-GTTTAAACGTCGACGATTGTAGTGTATTTTATGTT-3′ (KB3). The HeV G ORF was PCR amplified from plasmid pCP484 (HeV G gene in pFastBac1) (Wang *et al.*, 2000; Yu *et al.*, 1998) using primers 5′-GTTTAAACGTCGACCACCATGATGGCTGATTCCAAATTGGTAAGC-3′ (KB7) and 5′-GTTTAAACGTCGACCAATCAACTCTCTGAACATTGGGCAGGTATC-3′ (KB8). All PCR were done using Accupol DNA polymerase (PGS Scientifics Corp., Gaithersburg, MD) with the following settings: 94°C for 5 min initially and then 94°C for 1 min, 56°C for 2 min, 72°C for 3 min; 25 cycles. These primers generated PCR products for the HeV F and HeV G ORFs flanked by *Sal*I sites, with an additional *Pme*I site flanking each *Sal*I site, and the 5′ end of each gene possessed the sequence CACC upstream of the initial ATG. All PCR products were gel purified (Qiagen, Valencia, CA). After gel purification, HeV F and HeV G were subcloned into a TOPO vector (Invitrogen Corp., Carlsbad, CA). The TOPO constructs were then digested with *Sal*I and inserted into the *Sal*I site of pMCO2. All constructs were initially screened by restriction digestion and further verified by sequencing. The recombinant viruses were then obtained using standard techniques employing *tk* selection and *E. coli* β-glucuronidase (GUS) staining (Broder and Earl, 1999). Briefly, CV-1 cells were transfected with either pMCO2 HeV F or pMCO2 HeV G using a calcium phosphate transfection kit (Promega, Corp., Madison, WI). These monolayers were then infected with Western Reserve (WR) wild-type strain of vaccinia virus at a multiplicity of infection (m.o.i.) of 0.05 PFU/cell. After 2 days the cell pellets were collected as crude recombinant virus stocks. TK$^-$ cells were infected with the recombinant crude stocks in the presence of 25 μg/ml 5-bromo-2′-deoxyuridine (BrdU) (Calbiochem, La Jolla, CA). After 2 h the virus was replaced with an EMEM-10 overlay

containing 1% low-melting-point (LMP) agarose (Life Technologies, Gaithersburg, MD) and 25 μg/ml BrdU. After 2 days of incubation an additional EMEM-10 overlay containing 1% LMP agarose, 25 μg/ml BrdU, and 0.2 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Clontech, Palo Alto, CA) was added. Within 24–48 h blue plaques were evident, picked, and subjected to two more rounds of double-selection plaque purification. The recombinant vaccinia viruses vKB1 (HeV F) and vKB2 (HeV G) were then amplified and purified. Bacteriophage T7 RNA polymerase was produced by infection with vTF7-3, which contains the T7 RNA polymerase gene linked to a vaccinia virus promoter (Fuerst *et al.*, 1986). The *E. coli LacZ* gene linked to the T7 promoter was introduced into cells by infection with vaccinia virus recombinant vCB21R–*LacZ*, which was described previously (Alkhatib *et al.*, 1996a). For cell fusion assays, either we infected cells with the appropriate vaccinia virus encoding the HeV F or G or we transfected cell monolayers with the pMC02-based plasmid constructs containing these genes followed by infection 4 h later with WR vaccinia virus. Transfection of monolayers was performed with DOTAP (Roche Diagnostics Corp., Indianapolis, IN).

Cell–cell fusion assays

Fusion between HeV glycoprotein-expressing cells and target cells was measured by a reporter gene assay in which the cytoplasm of one cell population contained vaccinia virus-encoded T7 RNA polymerase and the cytoplasm of the other contained the *E. coli LacZ* gene linked to the T7 promoter; β-Gal is synthesized only in fused cells (Nussbaum *et al.*, 1994). Vaccinia virus-encoded proteins were produced by incubating infected cells at 31°C overnight (Berger *et al.*, 1995). Cell–cell fusion reactions were conducted with the various cell mixtures in 96-well plates at 37°C. Typically, the ratio of HeV glycoprotein-expressing cells to target cells was 1:1 (2×10^5 total cells per well, 0.2-ml total volume). Cytosine arabinoside (40 μg/ml) was added to the fusion reaction mixture to reduce nonspecific β-Gal production (Berger *et al.*, 1995). For quantitative analyses, Nonidet-P40 was added (0.5% final) at 2.5 h and aliquots of the lysates were assayed for β-Gal at ambient temperature with the substrate chlorophenol red-D-galactopyranoside (Roche Diagnostics Corp.). For inhibition by peptides, serial dilutions of peptides were performed and added to HeV glycoprotein-expressing effector cells immediately prior to the addition of target cell populations. For inhibition by HeV-specific antisera, serial dilutions of the various rabbit sera were performed and added to HeV glycoprotein-expressing effector cells just prior to the addition of target cells. All assays were performed in duplicate and fusion results were calculated and expressed as rates of β-Gal activity (change in optical density at 570 nm per minute × 1000) (Nussbaum *et al.*, 1994).

Peptide synthesis

The following hydrophilic peptide sequences were chosen for synthesis and immunization based on analysis of the hydrophobicity plots of the HeV F and G glycoproteins: CKGITRKYKIKSNPLTKDIVIK (F2) and CKSDSGDYNQKYIATKVERGKKDK (G1). Another 42-amino-acid peptide corresponding to the C-terminal α-helical heptad domain of HeV F was also synthesized, HQSIQTKVDISSQISSMNQSLQQSKDYIKEAQKILDTVNPSL (FC1). The sequence of peptide FC1 was based on the published sequence (Gould, 1996), which was later corrected (GenBank Accession No. AF017149) (Wang *et al.*, 2000), and the first 6 residues of the N-terminus of FC1 are irrelevant; however, this change is distant from the leucine zipper region and did not affect the peptide's activity. A control 44-amino-acid peptide derived from the cytoplasmic tail of the IL-2 receptor gamma chain protein, LERTMPRIPTLKNLEDLVTEYHGNFSAWSGVSKGLAESLQPDYS (IL2Rg), was used as the irrelevant control. Synthesis of each peptide was accomplished on an Applied Biosystems Model 433 Peptide Synthesizer using HBTU/HOBt activation on a hydroxmethiphenoxymethyl-copolystyrene-1% divinylbenzene resin. Upon synthesis completion, the resin was washed twice with dichloromethane (DCM) followed by three washes with methanol and allowed to dry. Cleavage of the peptide from the resin was obtained using Reagent R (90% trifluroacetic acid (TFA), 5% thioanisole, 3% 1,2-ethanedithiol, and 2% anisole) at room temperature for 3 h. The peptide was isolated from the mixture by vacuum filtration through a sintered glass funnel into cold ethyl ether, which permitted precipitation. Peptide and ether were transferred to a 50-ml centrifuge tube and centrifuged. The peptide pellet was resuspended in cold ether and centrifuged three separate times to remove residual scavengers and acid. Following the third wash the pellet was allowed to dry completely. Once dry, the peptide was resuspended in 95% water/5% CH_3CN (the pH was adjusted to ~7 using dilute NH_4OH), frozen at −20°C, and lyophilized.

Polyclonal antibodies

Peptide-specific rabbit sera to the HeV F and G glycoproteins were prepared with the Imject Maleimide Activated KLH Kit (Pierce, Rockford, IL). The F2 and G1 synthetic peptides were reduced with 250 μM dithiothreitol (DTT) (ICN Biomedicals, Cleveland, OH) for 1 h at 37°C prior to conjugation. Excess DTT was removed using 1800 MW gel exclusion columns (Pierce). Then 1.56 mg of recovered G1 was conjugated to 2 mg of activated KLH and 1.25 mg of recovered F2 was conjugated to 2 mg of activated KLH. Conjugates were purified

using a 5000 MW gel exclusion column (Pierce). Each peptide–KLH conjugate was stored in 200-μg aliquots at −80°C. Two hundred micrograms of F2-KLH conjugate containing 1× RIBI (RIBI Immunochem Research Inc., Hamilton, MT) in 1 ml PBS and 200 μg G1–KLH conjugate containing 1× RIBI in 1 ml PBS were administered independently to two rabbits as follows: 0.05 ml intradermal at six sites, 0.3 ml into each hind leg, and 0.1 ml subcutaneous in the neck region. Equivalent boosts were given on days 28, 56, and 84. Test bleeds were collected on day 35, and crop bleeds were collected on days 63 and 91. In addition, serum from a rabbit immunized with gamma-irradiated HeV was also used in some experiments.

Metabolic labeling and immunoprecipitation

For labeling of HeV glycoproteins expressed by recombinant vaccinia viruses, HeLa cells were infected at a m.o.i. of 10 PFU/cell. At 6 h postinfection, monolayers were washed, overlaid with methionine- and cysteine-free minimal essential medium (Life Technologies) containing 2.5% dialyzed fetal calf serum (Life Technologies) and 100 μCi of ^{35}S-labeled ProMix/ml (Amersham Pharmacia Biotech, Piscataway, NJ), and incubated overnight. Alternatively, a pulse–chase labeling procedure was performed where after 8 h of infection the cells were pulsed with 100 μCi of ^{35}S-labeled ProMix/ml for 15 min and then chased with a 200-fold excess of methionine and cysteine for 1 h. Lysis of cells was performed in 100 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.5% Triton X-100. Typically, 0.5–1.0 μl of antiserum or normal rabbit serum was utilized per immunoprecipitation. Incubations for at least 1 h at room temperature were followed by addition of Protein G–Sepharose for at least 30 min. Complexes were washed twice with lysis buffer (100 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.5% Triton X-100) and once with DOC buffer (100 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS). Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (10%) and visualized by autoradiography.

Western blot analysis

BSC-1 cell monolayers were infected overnight at a m.o.i. of 10 with vaccinia virus encoding wild-type HeV F or HeV G. Cells were extracted with 0.5% Triton X-100 in 100 mM Tris–HCl, pH 8.0, 100 mM NaCl and the nuclei were removed by centrifugation. Samples were prepared by boiling in sample buffer containing 2-mercaptoethanol. Extracts from 5 × 10^4 cells (total) were loaded per well onto a 10% SDS–PAGE gel. Following transfer to nitrocellulose paper, the blot was probed with either peptide-specific or virus-specific rabbit antiserum. The blot was then incubated with HRP-conjugated rabbit anti-rabbit IgG and developed with the SuperSignal chemiluminescence kit (Pierce).

ACKNOWLEDGMENTS

We thank Joseph Isaac for viruses and cells, Mike Flora for peptide synthesis, and Paul Selleck and Chris Morrissy for Hendra virus-specific rabbit antiserum. This study was supported by USUHS Grants R073HL and R073IL to C.C.B.

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Exhibit B

James Remenick

From: James Remenick
Sent: Thursday, June 21, 2007 2:52 PM
To: La Shaun Berrien (lberrien@hjf.org)
Cc: Marcia Daraban
Subject: FW: Request Virology Volume 290, Issue 1

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Sent: Friday, May 14, 2004 9:57 AM
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Alyssa - Great news! We have written confirmation from the publisher that the Virology artilce was not publicaly available until after the 13th, our filing date (e-mail see below). Woo hoo!!!

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Subject: FW: Request Virology Volume 290, Issue 1

Dear Mr. Walters,

This e-mail message will confirm that the article "Functional Expression and Membrane Fusion Tropism of the Envelope Glycoproteins of Hendra Virus," by Katharine N. Bossart, Lin-Fa Wang, Bryan T. Eaton, and Christopher C. Broder , appeared in *Virology*, Volume 290, Issue 1, 10 November 2001, pp. 121-135. The article was published online on November 15, 2001, and the printed issue was mailed to subscribers on November 20, 2001.

Sincerely yours,

Cindy MacDonald
Manager, Production Editorial Operations
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Hi, Andrea. We are interested in obtaining written confirmation of the date when the article listed below was available online, as well as the date that it was mailed to subscribers. The article was published in Virology Volume 290, Issue 1. Either an email or a written letter is fine. Thanks very much for all of your help, and let me know if you need anything else. Thanks!

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Functional Expression and Membrane Fusion Tropism of the Envelope Glycoproteins of Hendra Virus[*1]

Virology, Volume 290, Issue 1, 10 November 2001, Pages 121-135

Katharine N. Bossart, Lin-Fa Wang, Bryan T. Eaton and Christopher C. Broder